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Isolation of a calcium-binding peptide from tilapia scale protein hydrolysate and its calcium bioavailability in rats

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ABSTRACT

A calcium-binding peptide was successfully purified from tilapia (*Oreochromis niloticus*) scale protein hydrolysate by hydroxylapatite affinity (HA), gel filtration and reverse phase high-performance liquid chromatography (RP-HPLC). This peptide was sequenced as DGDDGEAGKIG (N- to C-terminal, MW = 1033 Da) based on MALDI-TOF-MS/MS. There was no significant difference in body weight gain, serum P content or alkaline phosphatase (ALP) level in Ca-deficient rats after supplementation with TSPH–Ca (tilapia scale protein hydrolysate calcium complex), CPP–Ca (casein phosphopeptide calcium complex), $CaCO_3$ or saline (control). However, the apparent absorptivity of calcium, serum and femur Ca content, femur bone mineral density and strength in the rats fed with TSPH–Ca were significantly higher than those of rats in the control and $CaCO_3$ groups ($P < 0.05$) and were similar to those from the CPP–Ca group ($P > 0.05$), indicating that TSPH possesses the potential to serve as an alternative for CPP.

1. Introduction

Calcium deficiency is widespread due to insufficient intake and diminished solubility of calcium caused by other constituents in food, such as phytates, cellulose and fat, among others (Reinhold, Lahimgarzadeh, Nasr, & Hedayati, 1973; Slavin & Marlett, 1980). The amount of Ca absorbed by the body is dependent upon the quantity of soluble calcium in the duodenum and proximal jejunum, where calcium remains in its soluble form at the alkaline pH and in the presence of calcium-binding peptides. (Fordtran & Locklear, 1966; Gleason, Grimme, Avioli, & Alpers, 1979). Various types of calcium-containing complexes and commercial products are available to treat and prevent calcium deficiency in humans, including calcium carbonate, calcium lactate and calcium gluconate. However, these calcium complexes have poor therapeutic effects in clinical practice, which is attributed to their low bioavailability and low concentration of calcium. Furthermore, calcium carbonate may lead to gastrointestinal side effects, including gas, flatulence and bloating (Straub, 2007). Casein phosphopeptides (CPP) have been observed by some investigators to enhance the absorption of calcium (Lee, Noguchi, & Naito, 1979; Sato, Noguchi, & Naito, 1986), and the main reason may be the increased solubility of calcium in the intestine by the formation of calcium complexes with divalent minerals. However, the relatively high price and complexity of producing CPP inevitably prevents its universal application as a calcium supplement. Consequently, exploiting novel calcium compositions based on other protein hydrolysates is an ideal alternative.

Nile tilapia (*Oreochromis niloticus*) is distributed worldwide, and its annual aquaculture production in 2010 was

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2.54 million tons according to the statistics from the Food and Agriculture Organization of the United Nations (Food and Agriculture Organization, 2012). The surface of the fish is fully covered with large scales, of which approximately 30% is made up of proteins comprising 18 of amino acids. Glycine (Gly), glutamic acid (Glu), hydroxyproline (Hyp), arginine (Arg) and aspartic acid (Asp), which are present in high proportions in fish scale proteins (Nagai, Izumi, & Ishii, 2004), can exhibit strong calcium binding affinities (Jung & Kim, 2007). However, the majority of the scales are dumped during fish processing, causing environmental pollution. Efficient utilization of fish scales would relieve the deficiency of animal protein to some extent and also provide collagen, peptide and calcium supplements for humans. Therefore, the objective of this study was to isolate and identify peptides with calcium-binding activity and to evaluate their calcium supplemental effects in Ca-deficiency rats.

2. Materials and methods

2.1. Materials

Nile Tilapia fish scales were obtained from Shandong Meijia Group (Rizhao, China). Pepsin (porcine gastric pepsin, activity 20 units mg^{-1} protein) was provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Trypsin (powder, porcine 1:250 > 250 USP units mg^{-1}), Flavourzyme (from *Aspergillus oryzae*, >20 units mg^{-1}), crystalline bovine albumin, and Sephadex G-15 were purchased from Sigma Chemical Co. (Sigma–Aldrich Inc., St. Louis, MO, USA). All other chemicals were of analytical reagent grade.

2.2. Preparation of fish scale protein hydrolysates

The pretreatments of fish scales were carried out according to the method of Zhang, Wang, and Xu (2009). Briefly, the fresh scales were stirred in 1.5 M NaCl solution for 24 h and then decalcified with 0.4 M HCl. The scales were washed with deionized water (conductivity approximately 1–1.5 μS cm^{-1}) and then dried and ground to powder (sieved through an 80 mesh sieve). The mixture of fish scale powder and deionized water (W_{powder}: W_{water}, 1:20) was hydrolyzed by pepsin (1.5%, W_{enzyme}: $W_{substrate}$, protein basis) at pH 1.8, 40 °C for 5 h. Then, 0.75% trypsin (w/w) was added for 5 h of hydrolysis, after which the pepsin was inactivated and the pH was neutralized to 8.0. The sample was boiled for 10 min and adjusted to pH 7.0, and then Flavourzyme (0.75%, w/w) was introduced for further hydrolysis at 50 °C for 5 h before heat-inactivation for 10 min at 100 °C. At various times during hydrolysis, an aliquot of hydrolysate was collected for determination of calcium-binding capacity. The mixture and collected aliquots were centrifuged at 3000*g* for 20 min at 4 °C and filtered through 0.45-μm Millipore filters to collect soluble peptides. The tilapia scale protein hydrolysates (TSPH) were freeze-dried for further analysis. The experiment was conducted in triplicate.

2.3. Degree of hydrolysis

The degree of hydrolysis (DH) of fish scale protein was monitored by reacting the free amino acid groups with O-phthaldialdehyde (OPA) in the presence of beta-mercaptoethanol. Then, the optimal density (OD) of the hydrolysate was assayed at 340 nm, as described by Nielsen, Petersen, and Dambamann (2001). DH was calculated as $(OD_{sample} - OD_{0h})/(OD_{total} - OD_{0h}) \times 100\%$. OD of hydrolysate at 0 h was used as a control (OD_{0h}). OD of hydrolysate completely hydrolyzed with 6 M HCl for 24 h at 120 °C was used as OD_{total}.

2.4. Isolation of calcium-binding peptides

The ceramic hydroxyapatite (type I, 40 μm, Bio-Rad, Hercules, CA, USA) was packed into a column (20 × 80 mm) and equilibrated with 10 column volumes (CVs) of buffer (10 mM sodium phosphate, pH 7.0). Demineralized fish scale protein hydrolysate was loaded into the HA column and incubated at 30 °C overnight. The unbound and weakly bound peptides were removed with the same buffer as above. Then, other fractions with high calcium-binding capacity were eluted separately with 100, 200 and 400 mM, pH 7.0 sodium phosphate buffer, and the last protein peak was collected. Afterwards, the sample was further separated with Sephadex G-15 beads (Pharmacia, Peapack, NJ, USA) on an open column (2.6 × 70 cm) at the flow rate of 0.7 mL min^{-1}. The component exhibiting the highest calcium-binding activity was applied to the RP-HPLC system equipped with a ZORBAX SB-C18 column (5 μm, 9.4 × 240 mm, Agilent Technologies, Santa Clara, CA, USA) for further separation. Mobile phases A and B were Milli-Q water and acetonitrile, respectively, each containing 0.1% trifluoroacetic acid (TFA). Linear gradient elution with a flow rate of 1.5 mL min^{-1} was performed for the first 30 min from 95/5 (A/B) to 84/16 before changing to 72/28 over the last 10 min. Fractions corresponding to major peaks were immediately collected and lyophilized. All of the chromatography processes were monitored at 220 nm. The crude protein content of fish scale powder was measured by Kjeldahl (1883) method. The protein/peptide content was determined by the Folin-phenol method of Lowry, Rosebrough, Farr, and Randall (1951).

2.5. Sequencing of the calcium-binding peptide

The lyophilized component (F312) was dissolved in 2 μL Millipore water (0.1% TFA) and added into matrix solution (5 mg/mL α-cyano-4-hydroxycinnamic acid, 0.1% TFA, 50% acetonitrile (ACN)). Then, 1 μL of the mixture was transferred onto a MALDI target plate and dried at room temperature, followed by mass spectrometric analysis. The instrument (4700 Proteomic Analyzer MALDI-TOF/TOF™, Applied Biosystems, Foster City, CA, USA) was equipped with a 335-nm pulsed nitrogen laser, and a delayed extraction source was performed under positive refraction mode. All spectra were recorded under signal averaging from 200 laser shots and external calibration. The peptide sequence was determined by the maximum identity using a Mascot search (http://www.matrixscience.com).